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(54) Title: ENHANCED PROTEIN THERMOSTABILITY AND TEMPERATURE RESISTANCE

(57) Abstract: Small heat shock proteins, e.g., *Pyrococcus fuiosus* (Pfu-sHSP), confer thermotolerance on cellular cultures and on proteins in cellular extracts during prolonged incubation at elevated temperature, demonstrating the ability to protect cellular proteins and maintain cellular viability under heat stress conditions. Such heat shock proteins are effective to combat enzymatic aggregation and intracellular precipitation during heat stress, and thereby enable enhancement of the utility and stability of enzymes in various applications, e. g., Taq polymerase in PCR applications, digestive enzymes in microbial degradative applications, etc.

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ENHANCED PROTEIN THERMOSTABILITY AND TEMPERATURE RESISTANCE

DESCRIPTION

Field of the Invention

The present invention generally relates to a heat shock protein from *Pyrococcus furiosus*, to a method of protecting and extending the durability of a recombinant DNA polymerase, and to a PCR kit.

Description of the Related Art

All organisms respond to elevated temperature by specifically inducing the expression of a set of new proteins, termed "heat shock proteins" or "HSPs." Although this response has been known for over thirty years, the specific role of individual HSPs in the overall response is still largely unknown. The HSPs to which functional character has been attributed have been characterized as molecular chaperones that enable protein folding, preventing denaturation of other proteins, or mediating proteolysis. This role, however, has only been demonstrated for a few of the many known HSPs and the function of other HSPs remains unknown. Moreover, it is not known which of the HSPs are essentials for the overall shock response except in the cases described below.

All organisms have a basal level of thermotolerance that is an organism-specific temperature threshold above which the organisms die. Basal levels of thermotolerance are probably determined by a variety of factors, including, for example, membrane composition and the innate thermal stability of enzymes involved in normal cellular processes. An additional

level of thermotolerance can be acquired by exposure of an organism to sublethal processes. Such "acquired thermotolerance" is believed to result from the production of HSPs in response to the sublethal high temperature exposure.

HSPs have been categorized by size and DNA sequence into families that are evolutionarily conserved. These families include the HSP 100, HSP 90, HSP 70, the HSP 60 and a variable class of low molecular weight proteins that range from 12-42 kDa. The HSPs found in this class of low molecular weight proteins are referred to as small heat shock proteins or sHSPs. In animals, this class of low molecular weight proteins ranges from 20-25 kDa. In the plant kingdom, the corresponding range is 14-20 kDa.

All of the low molecular weight HSPs are distinguished by conserved carboxy termini that are highly homologous to the α B-crystallin structural protein of the eye lens. α B-crystallin is itself capable of acting as a molecular chaperone, and all sHSPs have been demonstrated to exhibit chaperone activities in *in vitro* experiments. Their role in cells has not yet been demonstrated.

While it might be assumed that the HSPs play a role in thermotolerance because of the correlation of their abundant synthesis with exposure to increased temperature, earlier work with yeast had suggested that they are unimportant for the development of thermotolerance, as elimination of a single yeast sHSP had no effect on thermotolerance. In addition, in Drosophila cells, the use of antisense technology caused a specific decrease in the synthesis of the sHSP 26 protein, but such decrease had no effect on thermotolerance.

In addition to being induced by temperature stress, many HSPs, including those in the sHSP class, can be induced by other stresses such as exposure to arsenite, ethanol, heavy metals, amino acid analogs (Lee, Y.R., et al., Plant Physio. 110:241-48 (1996); and Nover, L., (ed.) Heat Shock Response, CRC Press (1990)) and water stress (Almoguera, C., et al., The Plant

Journal 4(6):947-58 (1993). In addition, increasing numbers of HSPs and HSP homologs are found to be regulated in developmental and tissue-specific ways (see, e.g., Almoguera, C. and J. Jordano, Plant Molecular Biol. 19:781-92 (1992); Apuya, N.R. and J.L. Zimmerman, The Plant Cell, 4:657-65 (1992); Cordewener, J.H.G., et al., Plant Cell 1:1137-1140 (1989). Proteins with highly conserved sequences related to HSPs, HSP cognates, may be expressed in non-stressed normal cells, but are not induced by thermal stress.

The mechanisms of action for the small HSPs are not clearly understood at present. There is a need for a better understanding of sHSPs despite other recombinant archaeal sHSPs that have been overexpressed in *E. coli*.

The present invention embodies an advance in the field of sHSPs that correlativey advances the understanding of the mechanism of sHSPs.

SUMMARY OF THE INVENTION

The invention relates to heat shock proteins and their methods of use.

In one aspect, the invention relates to a purified and isolated nucleic acid sequence encoding a heat shock protein comprising SEQ ID NO. 1.

Another aspect of the invention relates to the protein encoded by the nucleic acid comprising SEQ ID NO. 1, and to compositions comprising same.

Another aspect of the invention relates to a protein comprising the amino acid sequence of SEQ ID NO. 2, and to compositions comprising same.

A still further aspect of the invention relates to a method of protecting and extending the durability of a recombinant DNA polymerase, comprising the steps of:

purifying a low molecular weight heat shock protein;

adding the heat shock protein to a buffer solution containing the polymerase;

incubating the solution at extended temperature for extended time;

adding components necessary for PCR;

thermocycling the reaction to produce product from amplification of genomic deoxyribonucleic acid; and

examining the product of the reaction by gel electrophoresis.

Yet another aspect of the invention relates to a method of maintaining proteins in solution, comprising the steps of:

adding a low molecular weight heat shock protein to the solution;

elevating the temperature of the solution; and

measuring the enzymatic activity by absorbance.

A still further aspect of the invention relates to a PCR kit comprising the protein encoded by the nucleic acid comprising SEQ ID NO. 1, or the protein comprising the amino acid sequence of SEQ ID NO. 2, and one or more other PCR reagents.

In a further compositional aspect, the invention relates to a composition comprising (i) a biological component and (ii) an HSP or a precursor thereof, which is (A) exogenous to the biological component, and (B) thermostabilizingly effective for the biological component in the composition.

The invention contemplates in various further aspects:

a method of enhancing the stability of Taq polymerase in a PCR operation, by conducting the PCR operation in the presence of a HSP;

a PCR kit including PCR primers, Taq polymerase, deoxyribonucleoside triphosphates and an HSP;

transformed cells capable of expressing Pfu-sHSP;

recombinant DNA vectors for expression of Pfu-sHSP; and

a method of stabilizing a protein solution, including a first protein therein, against heat-mediated agglomeration of the first protein in the solution, by incorporating in the solution a heat shock protein that is non-endogenous with respect to the first protein.

Other aspects, features and embodiments of the invention will be more fully apparent from the ensuing disclosure and appended claims.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is the nucleotide sequence (SEQ ID NO. 1; GenBank Accession No. AF 256212) and the amino acid sequence (SEQ ID NO. 2; GenBank Accession No. AF 256212) of *Pyrococcus furiosus* heat shock protein.

Figure 2 demonstrates the SDS PAGE (15%) and blot analysis of Pfu-sHSP.

Figure 3 demonstrates the SDS PAGE (15%) analysis of thermal protection of *E. coli* crude extract by *Pyrococcus furiosus*-sHSP at 105°C.

Figure 4 is a graph of supernatent bovine glutamate dehydrogenase activity and A₂₈₀ values as a function of time.

Figure 5 demonstrates the agarose gel electrophoresis (1%) of the protection of Taq polymerase by the *Pyrococcus furiosus*-sHSP.

Figure 6 depicts an electrophoretic gel (1% agarose) showing the effect of Pfu-sHSP on Taq polymerase concentration in PCR reaction.

Figure 7 depicts another electrophoretic gel (1% agarose) showing the effect of Pfu-sHSP on Taq polymerase concentration in PCR reaction.

Figure 8 depicts yet another electrophoretic gel (1% agarose) showing the effect of Pfu-sHSP on Taq polymerase concentration in PCR reaction.

DETAILED DESCRIPTION AND PREFERRED EMBODIMENTS OF THE INVENTION

Relative to the present invention and its features, aspects and embodiments as more fully described hereinafter, the disclosure of U.S. Provisional Patent Application No. 60/60/197,274 is hereby incorporated herein by reference in its entirety.

Definitions

As used herein, the following terms have the following meanings.

As used herein, the term "heat shock protein" refers to any protein whose synthesis is enhanced when an organism or its cells are exposed to an increased temperature for that species; typically a temperature increase in a range of from about 5 to about 15°C.

As used herein, the term "low molecular weight heat shock protein" refers to those heat shock proteins that are between 12-42 kilodaltons (kDa) in size.

As used herein, the term "sHSP 20" refers to the small heat shock proteins encoded by SEQ ID NO. 1.

As used herein, the term "thermotolerance" refers to the ability of a cell to survive exposure to temperatures above its normal growth temperature.

As used herein, the term "basal thermotolerance" refers to the maximum temperature to which an organism or cell can survive when the shift to that temperature is rapid.

As used herein, the term "acquired thermotolerance" refers to the increase in thermotolerance that results from a prior (pre) exposure to a sublethal heat shock temperature.

As used herein, the term "transgenic cell line" or "transgenic culture" refers to a cell or culture that has stably incorporated added DNA sequences into its genome after deliberate introduction of DNA into that cell.

As used herein, the term "DNA molecule" refers to the polymeric form of deoxyribonucleotides (adenine, guanine, thymine or cytosine) in either single stranded form, or a double-stranded helix. This term refers only to the primary and secondary structure of the molecule, and does not limit it to any particular tertiary forms. Thus, this term includes double-stranded DNA found, inter alia, in linear DNA molecules (e.g., restriction fragments), viruses, plasmids and chromosomes.

As used herein, the term "vector" refers to a replicon, such as a plasmid, phage, cosmid or virus to which another DNA or RNA segment may be attached so as to bring about the replication of the attached segment. Specialized vectors were used herein, containing various promoters, polyadenylation signals, genes for selection, etc.

As used herein, the term "transcriptional and translational control sequences" refer to DNA regulatory sequences, such as promoters, enhancers, polyadenylation signals, terminators, and the like, that provide for the expression of a coding sequence in a host cell.

As used herein, the term "promotor sequence" is a DNA regulatory region capable of binding RNA polymerase in a cell and initiating transcription of a downstream (3' direction) coding sequence. Eukaryotic promoters will often, but not always, contain "TATA" boxes and "CAT" boxes. Prokaryotic promoters contain Shine-Dalgarno sequences in addition to the -10 and -35 consensus sequences.

As used herein, the term "selection gene" refers to a gene that enables the discrimination of cells displaying a required phenotype upon implementation of certain conditions. For example, the growth of bacteria in a medium containing antibiotics to select for the bacterial cells containing antibiotic resistance genes.

As used herein, the terms "restriction endonucleases" and "restriction enzymes" refer to bacterial enzymes , each of which cuts double-stranded DNA at or near a specific nucleotide sequence.

As used herein, the terms "transformed" or "transfected" by exogenous or heterogenous DNA when such DNA has been introduced inside the cell. The transforming DNA may or may not be integrated (covalently linked) into the genome of the cell. In prokaryotes, yeast and mammalian cells, for example, the transforming DNA may be maintained on an episomal element such as a plasmid. With respect to eukaryotic cells, a stably transformed cell is one in which the transforming DNA has become integrated into a chromosome so that it is inherited by daughter cells through chromosome replication. This stability is demonstrated by the ability of the eukaryotic cell to establish cell lines or clones of a population of daughter cells containing the transforming DNA.

As used herein, the term "clone" refers to a population of cells derived from a single cell or common ancestor by mitosis.

As used herein, the term "cell line" is a clone of a primary cell that is capable of stable growth *in vitro* for many generations.

In accordance with the present invention, conventional molecular biology, microbiology, and recombinant DNA techniques within the skill of the art. Such techniques are explained fully in the literature. See, e.g., Maniatis, Fritsch & Sambrook, "Molecular Cloning: A Laboratory Manual" (1982); "DNA Cloning: A Practical Approach," Volumes I and II (D.N. Glover, ed., 1985); "Oligonucleotide Synthesis" (M.J. Gait, ed., 1984); "Nucleic Acid Hybridization" (B.D. Hames & S.J. Higgins, eds., 1985); "Transcription and Translation" (B.D. Hames & S.J. Higgins, eds., 1984); "Animal Cell Culture" (R.I. Freshney, ed., 1986); "Immobilized Cells & Enzymes," IRL Press (1986); "A Practical Guide to Molecular Cloning," B. Perbal (1984).

In a further aspect of the invention, the reagents described herein can be packaged in a kit form for carrying out PCR. As used herein, the term "package" refers to a solid matrix or materials customarily utilized in such a kit system in the form of at least one or more enclosure that is capable of holding within fixed limits at least one or more of the reagent components for use in PCR. Such materials include glass and plastic (e.g., polyethylene, polypropylene, and polycarbonate) bottle, vials, paper, plastic, plastic-foil laminated envelopes and the like. Thus, for example, a package can be a glass vial used to contain the appropriate quantities of polynucleotide primer(s), genomic DNA, vectors and DNA polymerase or a combination thereof, in addition to an appropriate amount of sHSP. An aliquot of each component sufficient to perform at least one complete PCR procedure is provided in each package.

Kits useful for producing a primer extension product for amplification of a specific nucleic acid sequence using a primer extension reaction methodology also typically includes, in

separate container within the kit, dNTPs where N is adenine, thymine, guanine and cytosine and other like agents for performing primer extension reactions.

The reagent species of any system described herein can be provided in solution, as a liquid dispersion or as a substantively dry powder, e.g., the primers may be provided in lyophilized form.

The advantage of using a small heat shock protein to prevent the aggregation of mixtures of proteins is clear, but using a recombinant protein comprising a small heat shock protein was demonstrated by the inventors hereof to extend the half-life of a pure enzyme in vitro. At a temperature that was higher than the optimal temperature and Tmax of the enzyme, the inventors hereof demonstrated that the half-life of the enzyme incubated with the recombinant protein was increased approximately four-fold compared to the enzyme control alone.

The heat shock proteins of the invention, *Pyrococcus fuiosus* (Pfu-sHSP), confer thermotolerance on cellular cultures and on proteins in cellular extracts during prolonged incubation at elevated temperature, demonstrating the ability to protect cellular proteins and maintain cellular viability under heat stress conditions. Such heat shock proteins are effective to combat enzymatic aggregation and intracellular precipitation during heat stress, and thereby enable enhancement of the utility and stability of enzymes in various applications, e.g., Taq polymerase in PCR applications, digestive enzymes in microbial degradative applications, etc.

The cytoprotective character of the heat shock proteins of the invention also enables the commercial exploitation of correspondingly transformed cellular cultures for elevated temperature fermentation and microbial culturing operations that take advantage of improved kinetics associated with higher temperature processing regimes. Such cytoprotective

character further facilitates clinical applications in which heat-mediated undesirable side effects of pyrogenic therapeutic agents, thermal treatments and environmental exposure are combated by administration of heat shock proteins of the invention to a human or animal patient in need thereof (or by gene therapy producing in vivo expression of such cytoprotective proteins).

The present invention comprehends a composition comprising (i) a biological component and (ii) an HSP or a precursor thereof, which is (A) exogenous to the biological component, and (B) thermostabilizingly effective for the biological component in the composition.

The invention also contemplates enhancing the stability of Taq polymerase in a PCR operation, by conducting the PCR operation in the presence of a HSP. The PCR operation may be carried out with the provision of a PCR kit including PCR primers, Taq polymerase, deoxyribonucleoside triphosphates and an HSP according to the present invention.

Transformed cells capable of expressing Pfu-sHSP may be readily formed and utilized to produce Pfu-sHSP for various applications of the invention, e.g., by use of recombinant DNA vectors for expression of Pfu-sHSP. The resulting heat shock protein then may be employed for stabilizing a protein solution, including a first protein therein, against heat-mediated agglomeration of such first protein in the solution, by incorporating in the solution the Pfu-sHSP or other heat shock protein that is non-endogenous with respect to the first protein.

The small heat shock protein from *Pyrococcus furiosus* (Pfu-sHSP) is composed of 167 amino acid residues encoded by an ORF of 504 nucleotides (GenBank Accession number AF256212).

The invention is described more specifically hereinafter, in respect of the present inventors' work involving the gene encoding the small heat shock protein (sHSP) from the

hyperthermophile *Pyrococcus furiosus*, as cloned and overexpressed in *E. coli*. The sHSP gene was found to be inducible by heat shock at 105°C. In specific experiments, recombinant sHSP prevented the majority of *E. coli* proteins from aggregating in vitro for up to 40 minutes at 105°C, and also prevented bovine glutamate dehydrogenase from aggregating at 56°C. Survivability of *E. coli* overexpressing the sHSP was empirically determined to be enhanced approximately 6-fold during exposure to 50°C for 2 hours compared with a control culture which was not expressing sHSP. These results evidence the utility of heat shock proteins from hyperthermophiles in conferring a survival advantage on mesophilic bacteria by preventing protein aggregation at supraoptimal temperatures, and implicate usages of such heat shock proteins in microbial culturing, fermentation, and numerous other bioprocessing applications.

While the ensuing description is directed illustratively to small heat shock proteins (sHSP) of *Pyrococcus furiosus*, it is to be recognized that the utility of the present invention is not thus limited and that a wide variety of other sHSPs may be employed in accordance with the present invention. Examples include, without limitation, sHSPs from *P. horikoshii* and *Aquifex aeolicus*.

Accordingly, the features and advantages of the invention are more fully apparent from the following illustrative examples, which are not intended in any way to be limitingly construed, as regards the invention hereinafter claimed.

EXAMPLE 1

Cloning, Overexpression and Purification of the Recombinant Protein

The region encoding the Pfu-shsp gene (SEQ ID NO. 1; GenBank Accession No. AF 256212) (Figure 1) was amplified from *Pyrococcus furiosus* genomic DNA by PCR using two

primers: Pfu-shspN with an NcoI site (underlined) (*5'GCCATGGTGAGGAGAATAAGAAGATGG*) (SEQ ID NO. 3) and Pfu-shspC with an XhoI site (underlined) (*5'ACTCGAGCTATTCAACTTAACTTCGAATCCTTC*) (SEQ ID NO. 4).

The amplified ORF was cloned into the pCR Zero Blunt vector (Invitrogen, Carlsbad, CA). The insert was digested by NcoI and XhoI and then subcloned into the isopropyl- β -D-galacto-pyranoside (IPTG)-inducible pET19b expression vector (Novagen, Madison, WI) designated pPfu-shsp. The *E. coli* strain BL21(DE3) (Novagen, Madison, WI) carrying pSJS1240 which encodes rare tRNAs for leu and arg in *E. coli* (Kim et al. 1998d) was used as an expression host. *E. coli* cells carrying these 2 plasmids were grown in Luria-Bertani broth containing 50 μ g/ml ampicillin and spectinomycin to an A_{595} of 0.6.

The Pfu-sHSP expression was induced by 1mM isopropyl- β -D-galactopyranoside (IPTG) for 3 hours. The same strain carrying pET19b and pSJS1240 served as a control. SDS PAGE of *E. coli* overexpressing Pfu-sHSP crude extract reveals an additional protein of 20 kDa in size which corresponds to the protein molecular weight deduced from the predicted amino acid sequence. After induction, cells overexpressing Pfu-sHSP were harvested and resuspended in 25 mM potassium phosphate buffer, pH 7.0, 2mM DTT, 1 mM EDTA (buffer A). The cells were disrupted using a French Press (SLM instrument, Urbana, IL) at 16, 000 psi and centrifuged at 5000 $\times g$ for 15 minutes.

Pfu-sHSP appeared in a pellet fraction as indicated by SDS PAGE. The pellet then was dissolved in buffer A by heating at 85°C for 20 minutes. The dissolved pellet was filtered and loaded onto an anion exchange column (MonoQ, Pharmacia Biotech, Uppsala, Sweden) previously equilibrated with buffer A. The Pfu-sHSP was eluted using an NaCl gradient, at

0.35 M NaCl. The fractions were pooled and concentrated, and the protein appeared homogeneous by SDS PAGE electrophoresis visualized with silver stain.

Rabbit polyclonal antibody preparations against the Pfu-sHSP were obtained by immunization of a rabbit with the purified, recombinant Pfu-sHSP (Bio-world, Dublin, OH).

The Pfu-sHSP, containing 167 amino acid residues, was overexpressed in *E. coli*. The SDS PAGE revealed that the molecular weight monomeric Pfu-sHSP was 20 kDa. The amino acid sequence of this recombinant protein is shown in Figure 1 (SEQ ID NO. 2) (GenBank Accession No. AF256212).

The purified Pfu-sHSP was used throughout the experiments.

EXAMPLE 2

Induction of the Small Heat Shock Protein

Pyrococcus furiosus was grown as described elsewhere (Gonzalez et al., 1998) at 95°C for 3 hours and then shifted to 104°C for 2 hours. The cells were collected and frozen immediately and Western blot analysis was performed (Sambrook et al., 1989) to monitor the Pfu-sHSP induction.

The 104°C temperature is considered a heat shock temperature for *Pyrococcus furiosus* at which it grows optimally at 103°C. The control culture continued growing at 95°C without shifting to 104°C for another 2 hours. A strong signal was observed in the 2-hour heat shocked cells whereas there was no signal observed in the non-heat shocked culture. This indicated that native Pfu-sHSP is heat-inducible, and the protein is not synthesized at sub-inhibitory growth temperatures.

Figure 2 shows the SDS PAGE (15%) and the Western blot analysis of the recombinant and the native Psf-sHSP.

Specifically, *Pyrococcus furiosus* was cultured in a modified 20L fermentor (New Brunswick) or in with S⁰ in the medium without maltose (Adams, M.W.W. 1995. Large-scale growth of hyperthermophiles, p. 47-49. In F.T. Robb (ed.) and A.R. Place. *Archaea, a laboratory manual, thermophiles*. Cold Spring Harbor Laboratory Press, Plainview, NY). The cultures were incubated at 95°C for 4 hours, then shifted to 105°C for 0, 30, 60, 120 minutes before chilling on ice and harvesting by centrifugation at 7,500 x g for 15 minutes. The total protein of the cell extracts were measured and an equal amount of protein was loaded onto each lane of an SDS gel. Western blot analysis was conducted. A strong signal at 20 kDa was observed in lanes 2-4, corresponding to 30, 60, and 120 minutes after onset of heat shock whereas no signal was observed in lane 1 corresponding to the non-heat shocked control culture. This indicated that native Pfu-sHSP was strictly heat-inducible, and that protein was not required for rapid growth at the optimal growth temperature.

The expression of mRNA was measured from the gene encoding the Pfu-sHSP under heat shock conditions using Northern blot analysis. Total RNA was isolated from *P. furiosus* after exposure to 105°C for 120 minutes, compared to the non-heat shocked control culture. Total RNA (4μg) was electrophoresed on a 1.5% agarose gel for Northern blot analysis using the radiolabelled PCR probe generated by PCR amplification from primers Pfu-shspN and Pfu-shspC using ³²P labeled dCTP. Hybridization with this probe revealed a transcript of 600 nucleotides corresponding to the size of the putative Pfu-sHSP gene. A radiolabelled probe from the gene encoding *P. furiosus* glutamate dehydrogenase, which is expressed constitutively, was used as a control.

EXAMPLE 3

Protection of *E. coli* Proteins and Cell Viability Under Heat Stress

E. coli strains containing either pPfu-sHSP or pET19b were induced as described above. The total protection concentration was determined using the Bradford protein assay kit (BioRad, Hercules, CA). The total proteins in crude extract were diluted in buffer A to a concentration of 4 mg/ml. The crude extract of each sample was covered with mineral oil and heated at 105°C for 0, 20, 30 and 40 minutes. After cooling down at room temperature, the samples were centrifuged at 10,000 x g for 5 minutes and the supernatants were collected.

The soluble proteins were visualized by SDS PAGE (Sambrook et al., 1989).

The induced cells were also tested for survivability at 50°C. The cultures were shifted rapidly to 50°C in a water-bath shaker. Samples were taken at 0, 20, 40, 60 and 120 minutes. Samples of the culture were diluted at each time point and plated on Luria-Bertani agar containing 50µg/ml of ampicillin and spectinomycin. Cell viability was determined by counting the colony-forming units after overnight incubation.

The capacity for Pfu-sHSP to stabilize the full complement of soluble proteins in *E. coli* cell free extracts was determined. All of the proteins that can be detected by SDS PAGE in *E. coli* extracts containing overexpressed Pfu-sHSP remained soluble after heat treatment at 105°C, and were recovered in the supernatant fraction after centrifugation. The Bradford protein assay indicated that the total protein concentration in the supernatant of the control decreased approximately 50% whereas those in supernatant with the presence of Pfu-sHSP decreased only 5%.

Figure 3 shows the SDS PAGE (15%) analysis of the thermal protection of *E. coli* crude extract by Pfu-sHSP at 105°C.

As the Pfu-sHSP is capable of protecting E. coli proteins from aggregation in vitro, the issue of whether the Pfu-sHSP could protect cell viability was addressed. The E. coli culture overexpressing Pfu-sHSP and a control E. coli culture with pET vector and no insert were incubated at 50°C for 2 hours, while viability was measured. The single order death rate of E. coli overexpressing Pfu-sHSP was approximately 6-7 fold lower than that of the culture transformed with pET19b and pSJS1240. The difference in viability between protected and unprotected cells after 120 minutes at 50°C was approximately 3 orders of magnitude. See Table 1 in respect of the reduction of death rate at 50°C of the E. coli overexpressing Pfu-sHSP in vivo.

Table 1. Death rate of E. coli overexpressing Pfu-sHSP

E. coli BL21 (DE3)	Death rate (log CFU/100µl/min)
PET19b/pSJS1240	0.0058
PPfu-sHSP/pSJS1240	0.038

EXAMPLE 4

Glutamate Dehydrogenase Activity Assays

Bovine glutamate dehydrogenase (boGDH) (Sigma, Milwaukee, WI) was diluted in EPPS buffer, pH 7.5 to a concentration of 0.9 mg/ml to yield an initial rate of $\Delta A = 0.06/\text{minute}$. The enzyme was incubated at 56°C with 2.25 mg/ml of the purified Pfu-sHSP for various times. The samples were removed at 0, 2 and 4 minutes and then centrifuged at 10,000 x g

for 2 minutes. The supernatants were tested for the residual enzymatic activity following the method described previously (Robb et al., 1992). The residual activity of boGDH was determined using a Beckman DU640 spectrophotometer fitted with a temperature controller at 340 nm and 25°C. The assay mixture contained 100 mM EPPS pH 8.0, 65 mM glutamic acid and 16.25 mM NADP. There was no detectable GDH activity in the purified Pfu-sHSP.

The inventors hereof have found that the Pfu-sHSP can protect bovine GDH by aggregation prevention. As a small heat shock protein can prevent aggregation of mixtures of proteins, the inventors hereof tested the ability of the recombinant Pfu-sHSP to extend the half-life ($t_{1/2}$) of a pure enzyme in vitro. The mesophilic glutamate dehydrogenase from bovine (boGDH) (Sigma, Milwaukee, WI) was used as a model. The optimal temperature and T_{max} of boGDH are 25°C and 48°C, respectively. The purified Pfu-sHSP was used to protect boGDH under heat treatment at 56°C.

The control incubation was without Pfu-sHSP in the same buffer used to store the Pfu-sHSP. In addition, to confirm that the GDH activity was not from *E. coli* GDH that may possibly be a minor contaminant in the purified Pfu-sHSP resulting in increased GDH activity, the Pfu-sHSP preparation was assayed at high concentration for GDH activity using the same method. No GDH activity was detected in the purified Pfu-sHSP.

The $t_{1/2}$ of boGDH incubated with Pfu-sHSP at 56°C was increased approximately four-fold compared to the boGDH control. See Table 2 for the results.

Table 2. Specific activity of boGDH at 56°C

GDH	Half-life (seconds)
GDH (0.9 mg/ml)	38

GDH/Pfu-sHSP (2.25 mg/ml)	152
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The inventors hereof examined the possibility that the activity of boGDH was protected as a result of prevention of aggregation by Pfu-sHSP. The absorbance (A_{280}) of the supernatent of boGDH solutions incubated at 56°C with or without the presence of Pfu-sHSP was measured. The amount of precipitate had increased and the A_{280} of boGDH without Pfu-sHSP was reduced dramatically whereas that of boGDH with Pfu-sHSP was not.

In the experiment where boGDH was incubated alone, the apparent $t_{1/2}$ was approximately 2 minutes whereas the boGDH to which sHSP was added did not precipitate at all during the course of the experiment. The activity of the boGDH in the supernatants, on the other hand, declined in both cases, as shown in Figure 4. Thus, much of the boGDH that remained in solution was denatured. In this case, the enzyme was maintained in solution but not preserved from denaturation. This is an important result that indicates that the probable mode of action of Pfu-sHSP is preventing non-specific aggregation of proteins thereby allowing them to be recruited to either refolding or protein turnover pathways.

EXAMPLE 5

DNA Polymerase Protection by Recombinant Protein

1.25 Taq Polymerase (Sigma, catalog number D1806) was incubated with and without 7.5 μ g of Pfu-sHSP in 40 μ l of Taq polymerase buffer with 15 mM MgCl₂ (Sigma) at -10 and 50°C for 16 hours. PCR was done using the incubated Taq polymerase. The PCR reaction was carried out as follows:

1. 1x Taq polymerase buffer
2. 1 μ M forward and reverse primers
3. 0.2 mM dNTP
4. 1.25U Taq polymerase

The PCR cycles were carried out according to the following chart of cycles:

Cycles	1 cycle	28 cycles			1 cycle	
Temperature	94°C	94°C	52°C	72°C	52°C	72°C
Time	2 minutes	30 seconds	30 seconds	30 seconds	2 minutes	2 minutes

The PCR products were visualized in 1% agarose gel electrophoresis.

Taq polymerase was incubated with sHSP at 50°C for 20 hours. PCR of a target containing the gene encoding sHSP was performed using Pfu-sHSP primers and Pfu genomic DNA.

The results of the agarose gel electrophoresis showed the following:

Lane 1 100 bp ladder (Promega)

Lane 2 PCR with normal Taq polymerase

Lane 3 PCR with normal Taq polymerase and 1 μ l of sHSP (final concentration)

Lane 4, 5 PCR with Taq incubated at 50°C 20 hours with 1 μ l of dH₂O

Lane 6,7 PCR with Taq incubated with sHSP at 50°C 20 hours

Figure 5 shows the results of the agarose gel electrophoresis.

EXAMPLE 6

Limitation of the Polymerase Molecules to Carry Out PCR Products

This procedure is carried out by the following steps.

1. Taq polymerase is diluted into several dilutions.
2. The diluted Taq polymerase is incubated with and without small heat shock protein.
3. The PCR process is performed.
4. A comparison is made of the PCR products from Taq polymerase incubated with and without the sHSP to establish the lowest dilution that carries out the PCR products.

EXAMPLE 7

Limitation of the Polymerase Molecule to Sequence PCR Product

This procedure is carried out by the following steps.

1. Thermosequenase used for sequencing PCR products is diluted into several dilutions.

2. The enzyme from step 1 is incubated with and without sHSP.
3. The PCR process is performed.
4. An automated DNA sequencer is employed to carry out the sequencing.
5. The sequencing peaks as a result from the enzyme incubated with and without sHSP are compared.

EXAMPLE 8

Effect of Pfu-sHSP on Taq Polymerase Concentration in PCR Reaction

The effect of Pfu-sHSP on concentration of Taq polymerase (Fisher Scientific) in a PCR reaction medium was assessed in three separate experiments, whose results are shown in the electrophoretic gels depicted in Figures 6-8.

Figure 6 depicts an electrophoretic gel (1% agarose) showing the effect of Pfu-sHSP on Taq polymerase concentration in PCR reaction, at an sHSP concentration of 0.2 μ g/ μ l. The respective lanes 1-7 shown in the figure are as follows:

Lane 1	100 bp marker	
Lane 2	0.025 U/ μ l	+ sHSP
Lane 3	0.025 U/ μ l	- sHSP
Lane 4	0.005 U/ μ l	+ sHSP
Lane 5	0.005 U/ μ l	- sHSP

Lane 6 0.0025 U/ μ l + sHSP

Lane 7 0.0025 U/ μ l - sHSP

Figure 7 depicts another electrophoretic gel (1% agarose) showing the effect of Pfu-sHSP on Taq polymerase concentration in PCR reaction, at an sHSP concentration of 0.2 μ g/ μ l. The respective lanes 1-7 shown in the figure are as follows:

Lane 1 100 bp marker

Lane 2 0.025 U/ μ l + sHSP

Lane 3 0.025 U/ μ l - sHSP

Lane 4 0.005 U/ μ l + sHSP

Lane 5 0.005 U/ μ l - sHSP

Lane 6 0.0025 U/ μ l + sHSP

Lane 7 0.0025 U/ μ l - sHSP

Figure 8 depicts yet another electrophoretic gel (1% agarose) showing the effect of Pfu-sHSP on Taq polymerase concentration in PCR reaction, at an sHSP concentration of 0.2 μ g/ μ l. The respective lanes 1-7 shown in the figure are as follows:

Lane 1 100 bp marker

Lane 2 0.025 U/ μ l + sHSP

Lane 3 0.025 U/ μ l - sHSP

Lane 4 0.005 U/ μ l + sHSP

Lane 5	0.005 U/ μ l	- sHSP
Lane 6	0.0025 U/ μ l	+ sHSP
Lane 7	0.0025 U/ μ l	- sHSP

The results are consistent in the respective experimental runs, and show that the presence of Pfu-sHSP in the PCR reaction volume was associated with a higher concentration of Taq polymerase in the reaction medium than in the corresponding reaction volumes in which the Pfu-sHSP heat shock protein was not present.

The results thereby demonstrate the advantage of utilizing a heat shock protein in accordance with the invention, in PCR operations. The present invention contemplates a PCR kit including a heat shock protein component for use in the PCR reaction medium to resist aggregation and precipitation of the polymerase component of the reaction mixture. The PCR kit in a specific embodiment may therefore include PCR primers, Taq polymerase, deoxyribonucleoside triphosphates and an HSP. Alternatively, the PCR kit may include an HSP together with at least one other of the components specified in the preceding sentence.

The foregoing results evidence the utility of the inventive method for stabilizing a protein solution including a first protein, against heat-mediated agglomeration of such first protein in the solution, by incorporating in the solution a heat shock protein that is non-endogenous with respect to the first protein.

The clear effects of Pfu-sHSP on protein stabilization and the increased thermotolerance it confers on *E. coli* reflect the mechanisms of its action *in vivo*. Protein stabilization is consistent with the maintenance of solubility of proteins, thereby promoting refolding and

assembly. It is highly unexpected and fundamentally surprising that a component of the adaptive response of an archaeum growing at 100°C, such as *P. furiosus*, can enhance the heat resistance of organisms (e.g., *E. coli*) growing at much lower temperatures. The invention therefore embodies a substantial advance in the art of heat shock proteins, implicating a wide variety of applications in which such heat shock proteins confer enhanced thermotolerance, survivability and utility in the context of supraordinary thermal exposure conditions.

The disclosures of all references cited herein are hereby incorporated herein in their respective entirieties.

While the invention has been described herein with reference to various illustrative features, aspects, and embodiments, it will be appreciated that the utility of the invention is not thus limited, but rather extends to and encompasses other variations, modifications and other embodiments, as will readily suggest themselves to those of ordinary skill in the art. Accordingly, the invention is to be broadly interpreted and construed as including such other variations, modifications and other embodiments, within the spririt and scope of the invention as hereinafter claimed.

CLAIMS

1. A purified and isolated nucleic acid sequence encoding a heat shock protein comprising SEQ ID NO. 1.

2. The protein encoded by the nucleic acid comprising SEQ ID NO. 1.

3. A protein comprising the amino acid sequence of SEQ ID NO. 2.

4. A composition comprising the protein of claim 2.

5. A composition comprising the protein of claim 3.

6. A method of protecting and extending the durability of a recombinant DNA polymerase, comprising the steps of:
 - (a) adding a low molecular weight heat shock protein to a buffer solution containing said polymerase;

 - (b) incubating the solution at elevated temperature for a predetermined time;

 - (c) adding components necessary for polymerase chain reaction;

- (d) thermocycling said reaction to produce product from amplification of genomic deoxyribonucleic acid; and
- (e) examining said product by gel electrophoresis.

7. A method of maintaining proteins in solution, comprising the steps of:
 - (a) adding a low molecular weight heat shock protein to the solution;
 - (b) elevating the temperature of the solution; and
 - (c) measuring the enzymatic activity by absorbance.
8. A PCR kit, comprising a protein as in claim 2 and at least one other PCR reagent.
9. A PCR kit, comprising a protein as in claim 3 and at least one other PCR reagent.
10. A method of enhancing the stability of Taq polymerase in a PCR operation, comprising conducting said PCR operation in the presence of a HSP.
11. The method of claim 10, wherein said HSP comprises Pfu-sHSP.

12. The method of claim 10, wherein said HSP comprises a protein encoded by the nucleic acid comprising SEQ ID NO. 1.

13. The method of claim 10, wherein said HSP comprises a protein comprising the amino acid sequence of SEQ ID NO. 2.

14. A composition comprising (i) a biological component and (ii) an HSP or a precursor thereof, which is (A) exogenous to said biological component, and (B) thermostabilizingly effective for said biological component in the composition.

15. The composition of claim 14, wherein said biological component comprises a material selected from the group consisting of biological cells, and fractions and components thereof.

16. The composition of claim 14, wherein said biological component comprises an enzyme.

17. The composition of claim 14, wherein said biological component comprises a microbial culture.

18. The composition of claim 14, comprising a solution.
19. The composition of claim 18, wherein the biological component comprises a protein, and the HSP or precursor thereof is present in an effective amount to enhance resistance of the protein to aggregation and/or precipitation upon heat exposure of the composition.
20. The composition of claim 14, wherein said HSP comprises Pfu-sHSP.
21. The composition of claim 14, wherein said HSP comprises a protein encoded by the nucleic acid comprising SEQ ID NO. 1.
22. The composition of claim 14, wherein said HSP comprises a protein comprising the amino acid sequence of SEQ ID NO. 2.
23. A PCR kit including PCR primers, Taq polymerase, deoxyribonucleoside triphosphates and an HSP.
24. The PCR kit of claim 23, wherein said HSP comprises Pfu-sHSP.

25. The PCR kit of claim 23, wherein said HSP comprises a protein encoded by the nucleic acid comprising SEQ ID NO. 1.

26. The PCR kit of claim 23, wherein said HSP comprises a protein comprising the amino acid sequence of SEQ ID NO. 2.

27. A transformed cell capable of expressing Pfu-sHSP.

28. A method of stabilizing a protein solution, including a first protein therein, against heat-mediated agglomeration of said first protein in the solution, comprising incorporating in the solution a heat shock protein that is non-endogenous with respect to said first protein.

29. The method of claim 28, wherein said heat shock protein comprises Pfu-sHSP.

30. A recombinant DNA vector for expression of Pfu-sHSP.

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FIGURE 1

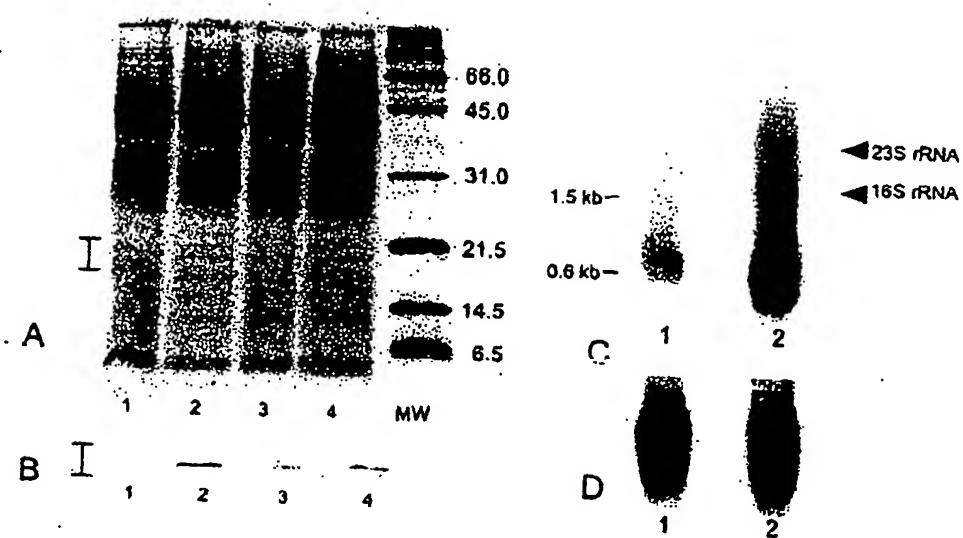


FIGURE 2

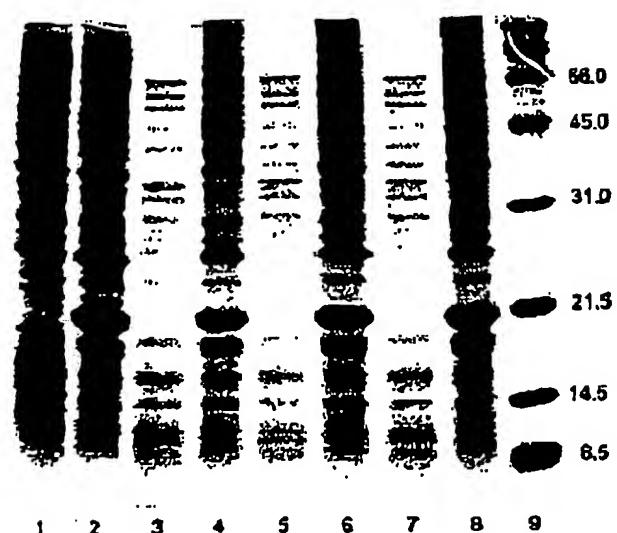


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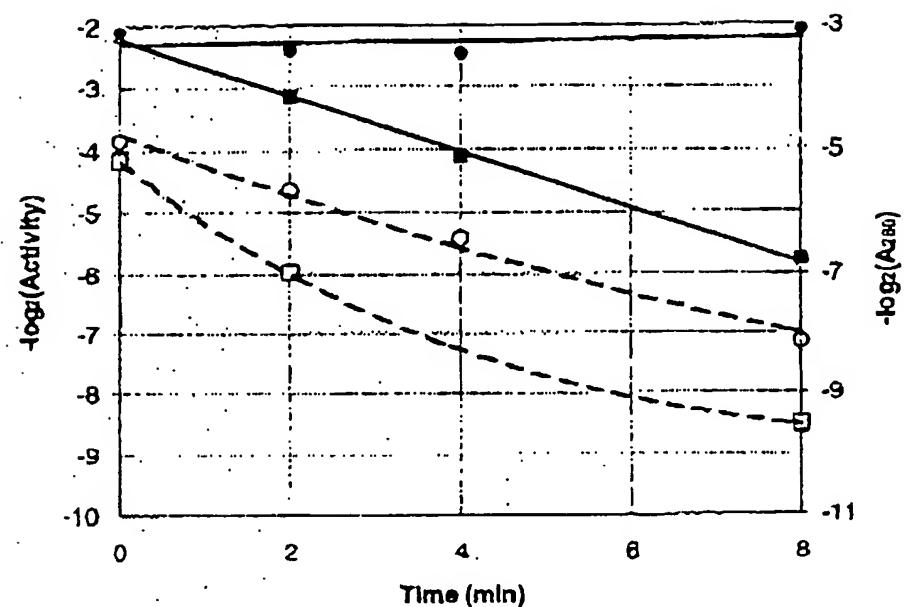


FIGURE 4

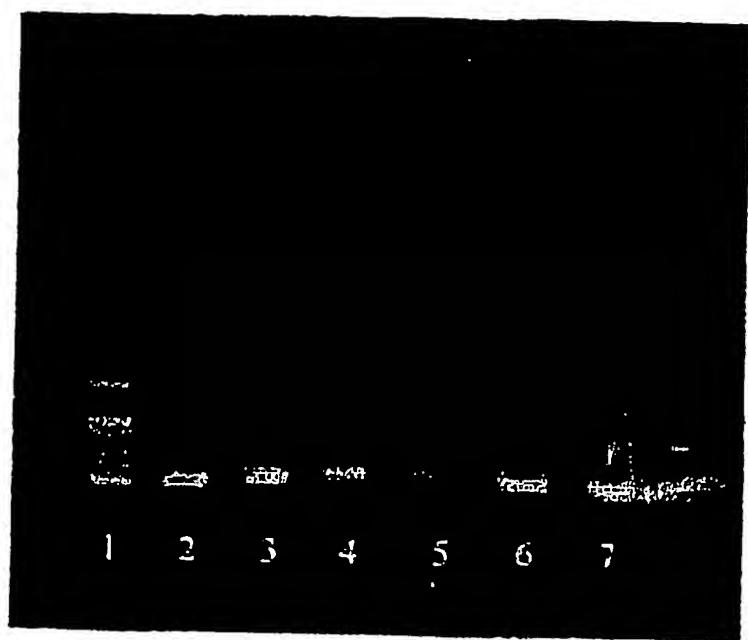


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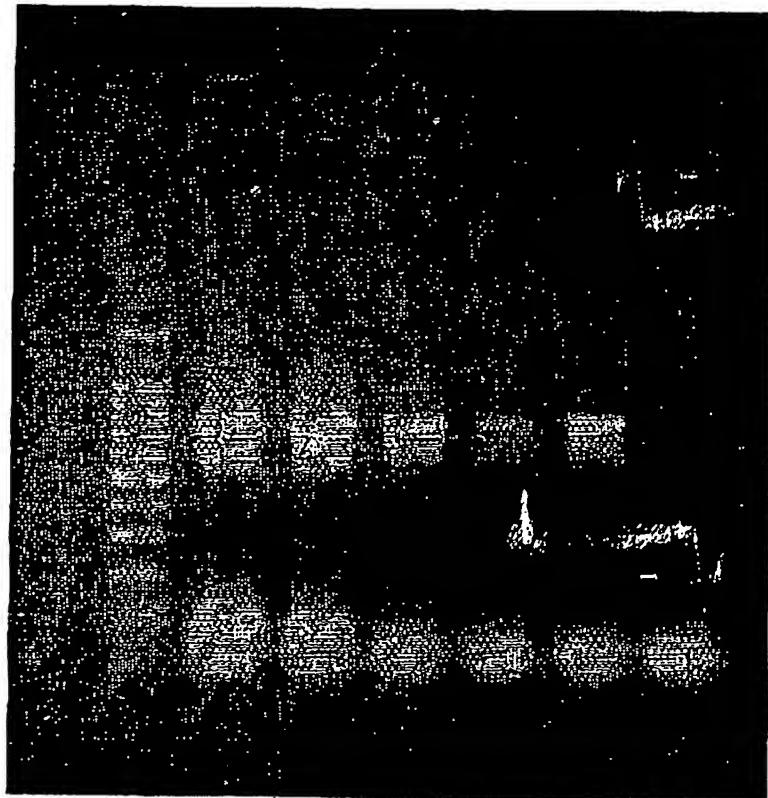


FIGURE 6

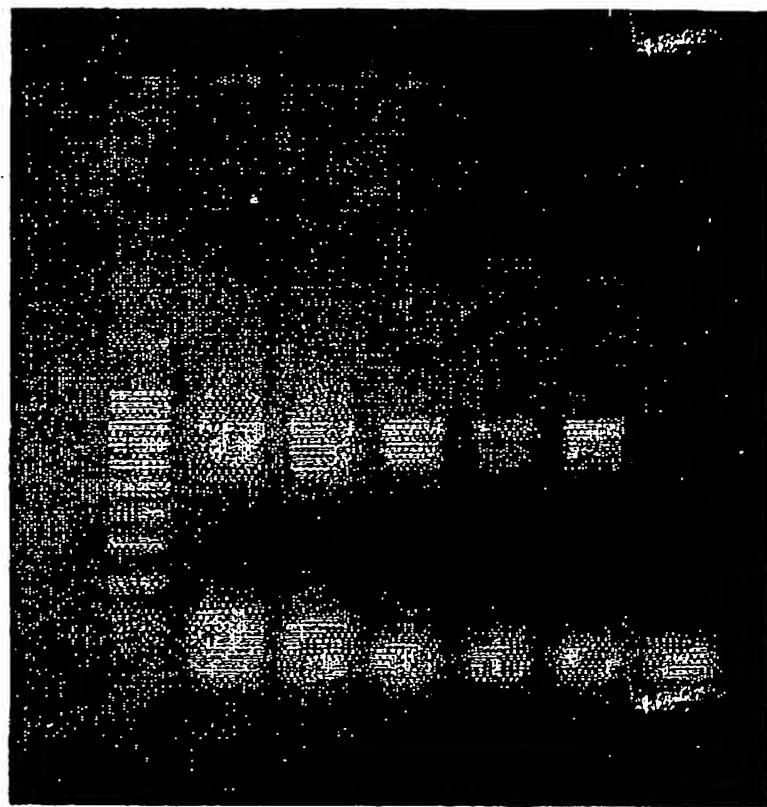


FIGURE 7

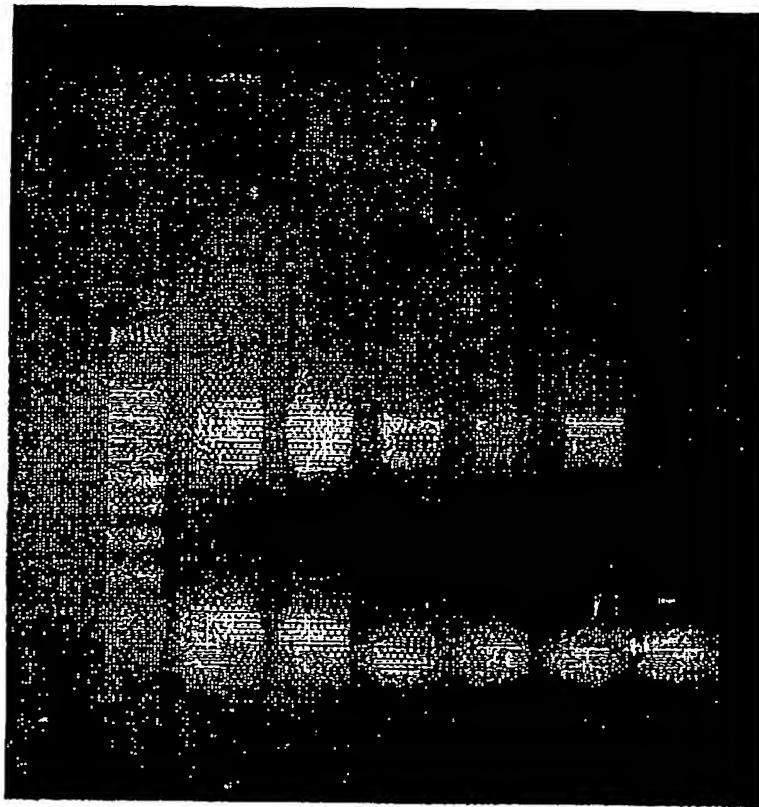


FIGURE 8

SEQUENCE LISTING

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Robb, Frank T.
Laksanalamai, Pongpan

<120> ENHANCED PROTEIN THERMOSTABILITY AND TEMPERATURE RESISTANCE

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Phe Asp Asn Gly Asp Glu Phe Val Ile Thr Ala Glu Leu Pro Gly Val
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INTERNATIONAL SEARCH REPORT

International application No.
PCT/US01/12290

A. CLASSIFICATION OF SUBJECT MATTER

IPC(7) : C07H 21/02; C12P 21/00, C12N 15/00,
US CL :Please See Extra Sheet.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 558/23.1, 24.5; 435/ 183, 188, 91.1, 91.2, 69.1, 70.1, 320.1, 325; 530/ 300

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Please See Extra Sheet.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US 5,474,892 A (JAKOB et al) 12 December 1995, entire document.	6-7, 10-11, 14-20, 23-24, 27-30

Further documents are listed in the continuation of Box C. See patent family annex.

"	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubt on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"Z"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search

30 JULY 2001

Date of mailing of the international search report

22 AUG 2001

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US CL :

536/23.1, 24.5; 435/ 189, 188, 91.1, 91.2, 69.1, 70.1, 320.1, 325; 530/ 300

B. FIELDS SEARCHED

Electronic data bases consulted (Name of data base and where practicable terms used):

Medline, Biosis, Embase, Sci Search, WPIDS, USPatfull

search terms: heat shock protein, stabilization of enzyme, Taq polymerase, stabilizing, stabilization of protein, Seq Id
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